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► **To cite this version:**

Reham Khalaf-Nazzal, Melissa A. Stouffer, Robert Olaso, Leila Muresan, Audrey Roumegous, et al.. Early born neurons are abnormally positioned in the doublecortin knockout hippocampus. *Human Molecular Genetics*, 2017, 26 (1), pp.90-108. 10.1093/hmg/ddw370 . hal-01512718

HAL Id: hal-01512718

<https://hal.sorbonne-universite.fr/hal-01512718v1>

Submitted on 24 Apr 2017

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Early born neurons are abnormally positioned in the doublecortin knockout hippocampus

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ABSTRACT

Human *doublecortin* (*DCX*) mutations are associated with severe brain malformations leading to aberrant neuron positioning (heterotopia), intellectual disability and epilepsy. The *Dcx* protein plays a key role in neuronal migration, and hippocampal pyramidal neurons in *Dcx* knockout (KO) mice are disorganized. The single CA3 pyramidal cell layer observed in wild type (WT) is present as two abnormal layers in the KO, and CA3 KO pyramidal neurons are more excitable than WT. *Dcx* KO mice also exhibit spontaneous epileptic activity originating in the hippocampus. It is unknown however, how hyperexcitability arises and why two CA3 layers are observed.

Transcriptome analyses were performed to search for perturbed postnatal gene expression, comparing *Dcx* KO CA3 pyramidal cell layers with WT. Gene expression changes common to both KO layers indicated mitochondria and Golgi apparatus anomalies, as well as increased cell stress. Intriguingly, gene expression analyses also suggested that the KO layers differ significantly from each other, particularly in terms of maturity. Layer-specific molecular markers and BrdU birthdating to mark the final positions of neurons born at distinct timepoints revealed inverted layering of the CA3 region in *Dcx* KO animals. Notably, many early-born 'outer boundary' neurons are located in an inner position in the *Dcx* KO CA3, superficial to other pyramidal neurons. This abnormal positioning likely affects cell morphology and connectivity, influencing network function. Dissecting this *Dcx* KO phenotype sheds light on coordinated developmental mechanisms of neuronal subpopulations, as well as gene expression patterns contributing to a bi-layered malformation associated with epilepsy.

INTRODUCTION

The development of neuronal circuits necessitates an intricate sequence of events including cell proliferation, migration, differentiation and synaptogenesis. The hippocampus is a laminated structure that develops following these steps, similar to the neocortex (1). There is evidence for birthdate-dependent layering of principal neurons with deep to superficial molecular and connectivity gradients (2-4). In the rodent, this pattern is induced from mid-embryogenesis, and newly born neurons carry intrinsic properties to help establish their adult phenotypes (5). A population of the earliest born neurons have specifically been shown to function as highly connected hub neurons in juvenile CA3 (6), helping to organize circuit development. Clonal analyses have also shown that related hippocampal pyramidal neurons derived from the same progenitor share interneuron connectivity (7). Thus, the timing and manner that hippocampal neurons are generated are critical for adult function.

Pyramidal cells of the major CA1 and CA3 fields are molecularly distinguishable (3, 8-10). Gene expression boundaries along the radial and septo-temporal axes distinguish hippocampal cell subpopulations related to anatomical position, birthdate and connectivity patterns (3, 5, 11). Subpopulations of interconnected neurons in different hippocampal fields have been found to share similar patterns of gene expression, birthdate and time window of synaptogenesis, these microcircuits being associated with hippocampal function (5, 12).

Mutations in genes associated with neurodevelopmental syndromes often reveal abnormal hippocampi in mouse models. This is characteristic of *doublecortin (Dcx)*, *Lis1*, and *alpha 1 tubulin (Tubal1)* mutations, which in human are associated with cortical malformations featuring neuronal disorganization, including lissencephaly and subcortical band heterotopia (SBH) that present clinically with varying degrees of intellectual disability (ID) and epilepsy (13-16). Mouse models show common but variable defects in hippocampal fields which are often disorganized (17-20). For unknown reasons, hippocampal defects appear to be a signature

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3 of type 1 lissencephaly mouse models, more so than neocortical abnormalities (21). Gene
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5 expression patterns that distinguish the features of abnormally positioned neurons have not yet
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7 been reported, which could shed light on their origins and functions.
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11 *Dcx* is a developmentally regulated microtubule associated protein (MAP) important for the
12
13 stabilization of microtubules in migrating and differentiating neurons (22-25). In *Dcx* KO mice,
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15 hippocampal pyramidal cells are abnormally positioned, mostly in the CA3 region, where the
16
17 single pyramidal cell layer observed in wild type (WT) is divided into two layers (18, 19). Video
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19 EEG recordings showed that *Dcx* KO mice have spontaneous epilepsy with the abnormal
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21 electrical activity originating in the hippocampus and secondarily propagating to the cortex
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23 (26). Extracellular and whole cell recordings in the *Dcx* KO CA3 region found that disorganized
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25 cells are more excitable than WT pyramidal cells (26, 27). Field recordings from *Dcx* KO brain
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27 slices suggested that pyramidal cells make a primary contribution to the spontaneous
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29 epileptiform activity, whereas inhibition remained strong during interictal events (27). It is
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31 hence important to further characterize *Dcx* KO CA3 pyramidal cells to investigate the
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33 mechanistic link between mutation of this protein, heterotopic cell positioning and
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35 hyperexcitability.
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43 In this study, we assessed global gene expression in the abnormal KO CA3 layers compared
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45 to the WT monolayer at postnatal day 0 (P0). At this stage of development, most CA3 neurons
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47 are expected to have arrived at their final destinations and to be growing axons and dendrites
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49 (2, 28). Gene expression changes at this stage may therefore contribute to abnormal morphology
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51 and circuitry seen in the mature brain (26, 27). We found that both KO layers have perturbed
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53 expression of genes indicating organelle abnormalities, increased cell stress and perturbed cell
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55 metabolism. Comparing KO layers also identified an unexpected inversion of a subpopulation
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57 of early-born KO neurons. Gene ontologies, layer-specific molecular markers and BrdU
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59 birthdating experiments confirmed transcriptome data suggesting that many of the most mature
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3 neurons are in an abnormal position and tissue environment at P0. Given the distinct molecular
4 identities and connectivity patterns conferred by birthdate, this perturbation likely influences
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6 neuron morphology and circuit formation, potentially contributing to the development of
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8 hyperexcitability in this mouse model.
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12 13 RESULTS

14 15 16 Laser capture microdissection (LCM) of CA3 cells in *Dcx* KO and WT

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18 In the *Dcx* KO hippocampus, the two abnormal bands of pyramidal cells in the CA3 region,
19 termed here *stratum pyramidale* internal (SPI, closest to the *stratum radiatum*) and *stratum*
20 *pyramidale* external (SPE, closest to the *stratum oriens*), are visible by late embryonic stages
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22 (Fig. 1). LCM at P0 allowed us to specifically separate SPI from SPE, and a similar region was
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24 excised from WT CA3 (Fig. 1). Reverse transcriptase (RT) PCR from LCM material confirmed
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26 that the microdissected samples expressed the CA3 marker *KAI* (*Grik4*) (9), but did not express
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28 the CA1 marker *Scip* nor the dentate gyrus marker *Prox1* (Supp. Fig. 1).
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35 Picochip analyses for RNA quality of CA3 LCM samples (n = 5 per condition) were
36 compared to analyses of RNAs from whole brain sections that had not been subjected to LCM.
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38 The latter had high RNA integrity number (RIN) values of 9 on average, indicating that the
39 sectioned and stained brain material was of good quality (Supp. Table 1). The LCM samples
40 displayed a lower RNA quality, which is expected from this procedure, with RIN values
41 between 5.2 and 7.2 (Supp. Fig. 2, Supp. Table 1). Overall, RNA integrity showed sufficient
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43 quality and reproducibility to proceed with microarray analyses.
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53 54 Differential gene expression between the WT CA3 region and *Dcx* KO SPI and SPE

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56 Reverse transcription, linear amplification and hybridization to microarrays were performed
57 according to Illumina protocols. Of the 25,600 well-annotated transcript probes on the *Illumina*
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59 *MouseRef-8 v2.0 Expression BeadChip* array, 11,800 (45%) on average demonstrated a
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3 detectable level of expression amongst the 15 samples (GEO accession GSE87019). After data
4 normalization (**Supp. Fig. 3**), 2361 and 1940 transcripts were found to be differentially
5 expressed in SPI and SPE cells respectively compared to WT when the P value cut-off was set
6 to < 0.01 , and 1758 and 1202 transcripts when $P < 0.005$ (**Fig. 2A, Supp. Table 2**). Considering
7 all SPI versus WT changes with a P value < 0.005 , an approximately equal number of transcripts
8 were up- (52 %) and down-regulated (48 %) (**Fig. 2A, Supp. Table 3A**). For SPE versus WT,
9 a greater proportion of transcripts were up-regulated (67 %), and only 33 % were down-
10 regulated (**Fig. 2A, Supp. Table 3A**). Considering genes showing a greater than 1.5 X change
11 compared to WT ($P < 0.005$), there were 49 up-regulated in SPI and 4 in SPE, and 11 down-
12 regulated in SPI and 3 in SPE (**Supp. Table 3B**). As expected, *Dcx* appeared amongst the most
13 significantly down-regulated in both SPI and SPE gene lists.
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30 Microarray analyses confirmed that WT and both KO cell populations expressed the
31 CA3 field-specific markers *KAI* (*Grik4*) (9) and *Bok* (29). Focusing on genes that were
32 commonly deregulated in both SPI and SPE KO cells compared to WT, 372 and 224 genes with
33 P values of < 0.01 and < 0.005 , respectively, were identified (**Supp. Table 4**). Some of these
34 common gene expression changes may be directly and specifically related to the inactivation of
35 *Dcx* (see below for genes coding protein partners), or due to deregulated cell processes. We
36 performed DAVID (Database for Annotation, Visualization and Integrated Discovery)
37 functional annotation clustering analyses for the 372 commonly differentially expressed genes
38 ($P < 0.01$; **Fig. 2B, Supp. Table 5A**). Significant categories include nucleotide binding and
39 translation, as well as Golgi apparatus and mitochondrion, the latter likely to indicate organelle
40 abnormalities, which indeed coincides with our previous observations by electron microscopy
41 (30). DNA repair and chaperone gene clusters also appeared as significantly different in both
42 KO layers compared to WT, suggesting abnormalities in cell stress responses.
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Indications for increased cell stress in *Dcx* KO layers at P0

Perturbed expression of genes related to organelles can be fundamentally linked with increased cell stress (31, 32). Expression of DNA repair proteins and upregulation of chaperones are known responses to oxidative stress that occur, for example, when the production of reactive oxygen species (ROS) is increased (33, 34). A significant number of genes involved in repairing DNA were found upregulated in SPI and SPE, including genes implicated in excision repair (*Ercc5*) and double-strand break repair (*Hmgb2*) (**Table 1**). Genes involved in protection against oxidative stress, including subunits of glutathione S-transferase (*Gstm1*, *Gstm5*) and sulfiredoxin (*Srxn1*), were also upregulated in SPI and/or SPE (**Table 1**).

Deregulated expression of heat shock protein (HSP) genes was also observed in both KO layers (**Table 1**). HSPs are highly conserved proteins that are often upregulated in response to several types of cell stress, functioning as molecular chaperones to help nascent protein folding or to refold damaged proteins. Two important HSPs upregulated in both SPE and SPI are *Hsp90ab1* and *Hspd1*. *Hsp90ab1* is located in the cytosol and interacts with protein kinases and transcription factors that have crucial roles in developmental processes involved in cell survival upon exposure to various stressful stimuli (34, 35). *Hspd1* (Hsp60) is a mitochondrial matrix chaperone, and deficits in this protein are associated with hypomyelination and neurodegenerative disorders (36). Also related to mitochondrial function, *Dnaja2*, upregulated in both SPE and SPI, functions as a co-chaperone of Hsp70s in protein folding and mitochondrial protein import (37). Other HSPs were conversely downregulated. For example, *Hspa8*, downregulated in SPI, is a constitutively expressed chaperone important for protein import into the endoplasmic reticulum and mitochondria, ubiquitin-proteasome degradation, autophagy, and disassembly of clathrin-coated vesicles (38). Also downregulated in SPI and SPE is *Herpud1*, a component of the endoplasmic reticulum quality control system involved in

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3 ubiquitin-dependent degradation of misfolded proteins (39). These proteins, related to organelle
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5 function, would be expected to contribute to cell stress when downregulated.
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8 Some changes in cell stress genes were specific to SPI. For example, pleiotrophin (*Ptn*)
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10 was up-regulated in SPI cells. It is a heparin-binding cytokine (40) that acts as a neuroprotective
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12 factor to prevent stress-induced cell death (41). The metallothioneins Mt1 (astrocytes >
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14 neurons) and Mt3 (brain specific isoform, neurons > astrocytes) were also upregulated only in
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16 SPI (**Table 1**). Mts are intracellular proteins that bind metals including zinc, and function in
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18 redox homeostasis (42). Mt overexpression may protect against apoptosis; zinc is a major
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20 inducer of its expression, as is oxidative stress and inflammation (42).
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24 Other indications of oxidative stress are related to proline metabolism. The
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26 mitochondrial matrix gene *Aldh4a1* (P5cd) (**Table 1**), critical for proline catabolism, is
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28 downregulated, and mutations in this gene in humans lead to hyperprolinemia, a disease
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30 associated with ID and epilepsy. Additionally, the proline transporter *Slc6a7* is upregulated in
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32 SPI (**Table 1**), further suggesting that intracellular proline could be increased. This Na⁺
33
34 dependent transporter is localized to synaptic vesicles and excitatory nerve terminals,
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36 corresponding to the proposed role of proline in ion channel and neurotransmitter receptor
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38 regulation (43). Proline metabolism is connected to the pentose phosphate pathway and redox
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40 homeostasis, and can also serve as a source of glutamate, ATP and ROS (44). On the other
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42 hand, proline might function as an antioxidant by decreasing ROS production (45). Thus several
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44 genes, some clearly linked to organelle function, are indicative of cell stress in P0 *Dcx* KO cells.
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53 **Metabolism genes are perturbed in KO layers**

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55 Potentially also linked to cell stress and/or organelle abnormalities, we observed several
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57 metabolic genes that are commonly deregulated in SPI and SPE compared to WT (**Table 2A**).
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59 For example, phosphofructokinase, platelet type (*Pfkp*), which catalyses the rate-limiting step
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3 in glycolysis, phosphoglycerate mutase 1 (*Pgam1*), another glycolytic enzyme, and hexokinase
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5 1 (*Hki*), an enzyme localized to the outer mitochondrial membrane that catalyzes the first step
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7 of glycolysis, are increased in both SPI and SPE. Genes involved in the Krebs cycle are
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9 upregulated in both layers including an ATP synthase subunit (*Atp5a1*), pyruvate
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11 dehydrogenase (*Pdhb*) and isocitrate dehydrogenase 3 (*Idh3g*). The latter enzyme has a role in
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13 redox homeostasis by generation of NADH/NADPH. On the other hand, there is a decrease in
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15 transaldolase 1 (*Taldo1*) in both SPI and SPE, an enzyme of the non-oxidative branch of the
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17 pentose phosphate pathway.
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22 Other notable changes in metabolism are related to lipids (**Table 2A**). Downregulation
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24 of genes related to lipid synthesis in SPI and SPE include ATP citrate lyase (*Acly*), acetoacetyl-
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26 CoA synthetase (*Aacs*) and phosphatidylglycerophosphate synthase 1 (*Pgs1*). Two enzymes
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28 involved in biosynthesis of cholesterol, mevalonate decarboxylase (*Mvd*) and hydroxysteroid
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30 17-beta dehydrogenase 7 (*Hsd17b7*), are also downregulated in both KO layers. Furthermore
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32 there is upregulation of three sphingomyelin phosphodiesterase genes coding for enzymes that
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34 break down sphingolipids (*Smpdl3a*, *Smpd4*, *Smpd1*), and upregulation of acyl-CoA
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36 thioesterase 1 (*Acot1*) that hydrolyses lipids (acyl-CoAs). Diazepam binding inhibitor (*Dbi*),
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38 also known as acyl-CoA binding protein, has been shown to regulate long-chain fatty acid
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40 metabolism, and is upregulated to a greater extent in SPI than SPE (46). Plasticity related
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42 protein 3 (*Plppr1*), downregulated in SPI, generates lipids important for cell signaling events
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44 including promotion of neurite growth (47). Overall, perturbed lipid metabolism may have
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46 numerous effects on cell signaling, function of membrane-bound organelles and particularly the
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48 plasma membrane of which cholesterol and sphingolipids are important components.
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58 **SPI layer cells are distinguishable from SPE layer cells**
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3 We next queried layer-specific gene expression. Many differentially expressed genes were
4 specific to either SPE versus WT, or SPI versus WT (**Supp. Fig. 4**). DAVID analyses to
5 compare these up- and down-regulated genes ($P < 0.005$) revealed that functional categories
6 were indeed different between SPE and SPI (**Supp. Table 5B-E**). The major categories with
7 the highest enrichment scores are summarized in **Fig. 3**. For SPI up-regulated genes, enriched
8 categories included several related to synaptic function, cell adhesion and metabolism. Some
9 of the latter genes were identified in previous analyses (**Table 2B**). Concerning SPE up-
10 regulated genes, enriched categories included organelle lumen, RNA processing and
11 mitochondrion. SPE down-regulated genes included membrane, GTP binding and actin
12 binding, whereas SPI down-regulated gene categories corresponded to those upregulated in
13 SPE (e.g., RNA processing, organelle lumen). These analyses hence suggest that the SPI layer
14 of cells differs substantially from SPE at P0.
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32 These findings are also supported by Ingenuity Pathway Analyses (IPA), focusing on
33 processes involved in central nervous system (CNS) development. For genes enriched in SPE
34 compared to WT, the broad categories predicted to be the most significantly activated (z-score
35 > 2) included proliferation of cells, movement of brain cells and development of CNS. For
36 genes enriched in SPI compared to WT, more than 15 categories were identified as being
37 significantly activated. The most significant cellular processes included formation of vesicles,
38 formation of neurites, tubulation of cells, cell movement, long-term potentiation of synapse and
39 formation of axons (**Supp. Table 6**).
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50 We next compared SPI and SPE to each other, and identified 2646 transcripts that were
51 significantly differentially expressed between these two *Dcx* KO cell populations (**Fig. 2A**;
52 **Supp. Table 4A**). Of these, 97 transcripts showed more than 1.5 X fold change difference ($P <$
53 0.005). The DAVID functional annotation clustering to compare enriched genes in SPI versus
54 SPE confirmed previous findings of comparing either to WT. Notably, genes preferentially
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3 enriched in SPI compared to SPE clustered in processes related to synapses, vesicular transport,
4 cell adhesion, glycoproteins and cytoskeletal organization (extended list shown in **Supp. Table**
5 **5F, Supp. Fig. 5A**). SPE gene expression compared to SPI on the other hand, showed clustering
6 enrichment related to organelle lumen, RNA processing, transcription and chromatin
7 modification (**Supp. Table 5G, Supp. Fig. 5B**). These clustering analyses are mostly exclusive,
8 and point to a difference in the maturity status between the two *Dcx* KO CA3 layers. Indeed, a
9 previous transcriptome study of several mouse lines showed that genes related to transcription
10 and translation are highly expressed during prenatal development, being turned down
11 progressively at P0 and P14, whereas expression of genes related to synaptogenesis showed the
12 opposite regulatory pattern (48). Therefore, based on functional annotation clustering of
13 differentially regulated genes, SPI cells appear to be more mature than SPE at P0.
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32 **Neuronal development genes are differentially expressed between SPI and SPE cells**

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34 *Dcx* and many other migration genes continue to be expressed postnatally; therefore, we
35 compared genes coding for *Dcx* partners and other genes known to be involved in migration
36 and/or differentiation. Genes potentially related to *Dcx*'s function that showed expression
37 differences were regulatory subunits of *protein phosphatases* 1 and 2 (either up or down, a
38 larger number deregulated in SPE) and the close homolog of *Dcx*, *Dclk1* (down in SPE) (**Table**
39 **3**) (23, 49). *Dclk2*, on the other hand showed no changes. Also down-regulated in SPI compared
40 to WT, were *Cdk5* and *Mark2*, both kinases that phosphorylate *Dcx* (50, 51), and AP1 (*Ap1m1*)
41 and AP2 (*Ap2m1*) mu subunit 1 genes of adaptor complexes, which interact with *Dcx* for vesicle
42 trafficking (52). *Kif1a*, a kinesin shown to be involved with *Dcx* in trafficking cargoes (53) and
43 *Nfasc*, coding for a cell adhesion molecule of the L1-CAM family interacting with *Dcx*, were
44 upregulated in SPI (54). If SPI cells are more mature, these gene differences may potentially
45 distinguish migration from differentiation roles of these partners.
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3 Several genes known to be involved in development of the cortex and hippocampus,
4 including *Nde1*, *Dab1* and *Kif2a* (55-57) were up-regulated in either SPI (*Nde1*, *Kif2a*), or both
5 SPI and SPE when compared to WT (**Table 3**). Three markers for interneurons (*Gad1*, *Sst*,
6 *Dlx1*) were shown also to be upregulated in both SPI and SPE, which may reflect some
7 interneuron disorganization in the KO (30).
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15 Through IPA functional clustering (e.g., **Supp. Table 6** 'Cellular movement'
16 categories), as well as literature searches, we assessed other genes known to be particularly
17 important for migration (**Table 3**). Notably, SPI showed a significant down-regulation of *Srf*, a
18 transcription factor regulated by changes in the ratio of polymerized to unpolymerized actin
19 that is important for neuronal migration (58), and alpha-N-acetyl-neuraminide alpha-2,8-
20 sialyltransferase 2 (*St8sia2*), a gene which is highly expressed in migrating neurons, but down-
21 regulated during neuronal differentiation (59). Furthermore, the Rho-GTPase *Rnd2*, a
22 modulator of radial migration in the developing cortex with a very specific spatiotemporal
23 expression pattern, being strongly expressed in radial glial progenitors and radially migrating
24 neurons but sharply down-regulated once neurons finish migration and start differentiation (60),
25 was similarly down-regulated in SPI.
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41 Thus, although the two *Dcx* KO populations share a common deficit in *Dcx*, and both
42 differ from WT, they show variable differences in expression of neuronal migration and
43 differentiation genes. These data may be consistent with the fact that SPI cells are potentially
44 already in the process of down-regulating a certain number of migration genes, and represent a
45 subpopulation more advanced in maturity than SPE cells.
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53 Further supporting this, changes in genes related to synaptogenesis, ion channels and
54 neurotransmission are upregulated in SPI (**Table 4**). Some mutant forms of these genes are
55 known genetic causes of epilepsy (e.g., *Sv2a*, *Lgi1* (61)). In addition, three genes encoding
56 subunits of the Na⁺/K⁺-ATPase cation transporter (*Atp1a2*, *b1*, *b2*) are upregulated in SPI.
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3 These plasma membrane channels regulate excitability by maintaining the resting potential, and
4 are also a major consumer of the cell's total energy (62). The up-regulation of all of these genes
5 may point to the advanced maturity of SPI cells. Finally, three categories related to blood vessel
6 development appeared in the functional annotation clustering for upregulated genes in SPI
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These plasma membrane channels regulate excitability by maintaining the resting potential, and are also a major consumer of the cell's total energy (62). The up-regulation of all of these genes may point to the advanced maturity of SPI cells. Finally, three categories related to blood vessel development appeared in the functional annotation clustering for upregulated genes in SPI (**Supp. Table 6**), perhaps also suggesting advanced maturity because neurons and blood vessels develop together (63).

A partial inversion of the *Dcx* KO hippocampal CA3 field along its radial axis

The hippocampus is made up of morphologically and functionally distinct pyramidal cells along its radial (deep to superficial), and septal to temporal axes. Molecular differences along these axes may contribute to the acquisition of specific phenotypes (3). We next analysed previously identified layer-specific markers along the radial axis in the *Dcx* KO, since neurons in radial layers are likely to have different maturities at P0, depending on their birthdates. Thompson et al (2008) described 45 molecular markers that specifically label CA3 outer boundary (deep, closest to the *stratum oriens*) neurons in the adult brain. We searched for these molecular markers in our P0 dataset and notably, found 13 genes to be significantly up-regulated in the SPI gene list (**Table 5A**). This suggests that some cells, which would have normally been closest to the *stratum oriens*, are positioned in much more superficial regions closest to the *stratum radiatum* of the *Dcx* KO hippocampus. Significantly, 7 of the 14 genes (*Sema5a*, *Fxyd5*, *Epha7*, *Kcnf1*, *Slc30a3*, *Fxyd7* and *Cdh13*) code for membrane proteins, which may be related to a role forming a boundary at the edge of the pyramidal cell layer. Two further genes down-regulated in outer-boundary cells (*Efnb3*, *Cadps2*) were also lower in expression in SPI than SPE.

Early-born subpopulation markers were likewise identified by Deguchi et al (2011), analysing adult hippocampal cell populations from *Lsi1* and *Lsi2* reporter mice. Despite the age

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3 and preparation differences of the starting samples, once again, we identified some genes in
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5 common in our SPI gene lists (**Table 5B**, 6 genes similarly upregulated in SPI vs. WT, of 53;
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7 6 genes similarly down-regulated of 77). Linking the datasets, *Suppression of tumorigenicity*
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9 *18 (St18)* and *stAR-Related Lipid Transfer (START) Domain Containing 13 (Stard13)* are
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11 commonly enriched in SPI, outer boundary (3) and early-born (5) datasets. These comparisons
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13 together indicate that many SPI cells may be misplaced, early-born, outer-boundary cells.
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17 The SPI-enriched expression of three of these genes, *St18*, *Cadherin 13 (Cdh13)* and *N-*
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19 *terminal EF-hand calcium binding protein 2 (Necab2)*, was confirmed by qPCR and/or *in situ*
20
21 hybridization experiments at P0 (**Fig. 4A,B, Supp. Table 7**). Furthermore, in the adult, *St18*,
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23 *Necab2* and *collagen type VI alpha 1 (Col6a1)*, the latter being an adult CA2/CA3 outer
24
25 boundary marker in WT (3, 64), were each found enriched in SPI cells, especially in rostro-
26
27 dorsal hippocampal regions (**Fig. 4C**). Outer boundary SPE cells were also labelled in more
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29 temporal regions.
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33 To better identify cells, we further checked by qPCR other genes that were differentially
34
35 regulated in SPI and/or SPE versus WT transcriptome data (**Supp. Table 3**). Notably, whereas
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37 some genes are specifically upregulated in association with SPI (*Dbi*, *Slc6a7*, *Camk2a*, *Necab2*,
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39 *Npy*, *Atp1b1*), *Adreno-receptor alpha 2A (Adra2a)* and *Gastrin-releasing peptide (Grp)*, were
40
41 enriched specifically in SPE (**Fig. 4A**). *In situ* hybridisations of *Grp*, a CA3 gene involved in
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43 cell migration, showed homogenous labelling in the WT CA3 region at P0, as well as the
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45 intermediate migration zone (**Fig. 4B**) (65, 66). In the *Dcx* KO, SPE cells are strongly labelled,
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47 with little expression seen in the SPI layer. We also confirmed that *Cholecystinin B receptor*
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49 (*Cckbr*) and *Cadherin 13 (Cdh13)* were similarly upregulated in SPI and downregulated in SPE
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51 (**Fig. 4A**), which may point to *Cckbr* having an outer-boundary profile.
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55 The SPI findings in particular strongly suggest that some cells destined for the outer
56
57 boundary layer of the CA3 pyramidal cell layer in the *stratum oriens* region, potentially born
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3 at early stages of CA3 neurogenesis (2), are abnormally, superficially positioned in the *Dcx* KO
4 SPI with respect to SPE. SPE on the other hand, may resemble more generally the WT
5 pyramidal cell layer organisation, containing CA3 cells born at later time-points.
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10 The similar neurodevelopmental outcomes of other hippocampal heterotopia mouse
11 models (*Lis1*, *Tuba1a*) suggest that the molecular mechanisms mediating these phenotypes may
12 overlap, at least in the CA3 field. To further investigate this possibility, we performed *in situ*
13 hybridizations in the *Tuba1a* (+/S140G) mutant mouse at P0. *St18* and *Necab2*, which were
14 found predominantly in the *Dcx* KO SPI layer (**Fig 4**), also primarily label inner-layer cells in
15 the CA3 region in the *Tuba1a* mutant compared to a control littermate (**Supp Fig. 6**).
16
17 Furthermore, *Grp* expression is similar between *Tuba1a* and *Dcx* mutants, with relatively
18 homogenous labeling across the major CA3 layer and the migrating cell layer, however with
19 decreased labeling in the inner-most cells in the mutant. This is intriguing because similar
20 positioning of early-born CA3 cells across several mouse mutant lines might indicate that these
21 neurons migrate normally and are less susceptible to cytoskeletal-related gene mutations,
22 perhaps indicating that migration modes/mechanisms are different between early- and late-born
23 neurons in this region.
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43 **BrdU birthdating experiments to label early- and late-born cells**

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45 Perturbation of layer-specific marker data discussed above suggested that the inside-out
46 layering of the CA3 region of the hippocampus is abnormal in *Dcx* KO mice. To study cell
47 birthdates in more detail, bromodeoxyuridine (BrdU) was injected in *Dcx* (+/-) pregnant dams
48 at either E12.5 or E16.5 to label early-born and late-born neurons, respectively, and brains from
49 the exposed embryos were analysed at P21-24 to determine the final destinations of CA3 cells.
50
51 In WTs, injection of BrdU at E12.5 labelled many neurons (NeuN+) along the border of the
52 pyramidal cell layer and *stratum oriens*, as expected (2, 5), with 67 +/- 1.3 % of total BrdU-
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3 labelled neurons found in the outer half of the CA3 *stratum pyramidale* and the other 33 +/- 1.3
4
5 % in the inner half ($P < 0.001$, paired t -tests, $n = 4$ mice; **Fig. 5**). When looking at each of the
6
7 CA3 subfields separately, CA3a and b showed a greater adherence to inside-out labelling
8
9 compared to CA3c, in which there was no significant difference between the percentages of
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11 BrdU+ neurons located in the inner or outer halves. By contrast, 56 +/- 1.7% of BrdU-labelled
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13 neurons were found in the SPI of *Dcx* KO mice, with the remaining 46% in SPE ($P < 0.05$,
14
15 paired t -test, $n = 4$ mice; **Fig. 5**). Comparing the CA3 subfields in KO and WT, the majority of
16
17 the lamination defect appears to be in CA3a and b. Overall, the proportion of BrdU+ neurons
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19 labelled at E12.5 in the inner portion of CA3 is greater in KO vs. WT mice ($P < 0.001$, Chi-
20
21 squared test).
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27 Injection of BrdU at E16.5 is expected to label late-born neurons that settle superficially
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29 in the CA3 close to the *stratum radiatum* (2, 5). In the WT CA3, 72 +/- 1.6% of BrdU+ cells
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31 were detected in the inner portion of the pyramidal cell layer ($P < 0.001$, paired t -test, $n = 4$
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33 mice; **Fig. 5**). Once again, the CA3a and CA3b subfields showed greater adherence to inside-
34
35 out labelling, with CA3c actually having more BrdU+ neurons in the inner portion of the
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37 pyramidal layer ($P < 0.05$, paired t -test, $n = 4$ mice; **Fig. 5**). In the *Dcx* KO CA3 region, BrdU+
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39 neurons were mainly located in the outer (SPE) layer (82 +/- 1.4%, $P < 0.001$, paired t -test, $n =$
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41 5 mice; **Fig. 5**). There were significantly more BrdU+ neurons in SPE in each CA3 subregion,
42
43 even in CA3c. Relative to SPE itself, many of these late-born BrdU+ neurons appeared to be
44
45 correctly placed in the internal portion of CA3a and b. These data hence confirm a partial
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47 inversion of birthdate-dependent, inside-out layering, predicted from the transcriptome data,
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49 affecting early-born, outer boundary cells, found in an abnormal superficial position in the *Dcx*
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51 KO hippocampus.
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DISCUSSION

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3 In the present study, we queried the genetic content and origin of abnormally positioned
4 hippocampal neurons in a mouse model of type 1 lissencephaly associated with epilepsy. To
5 our knowledge, no other studies have attempted to assess global gene expression in heterotopic
6 neurons. We questioned how the dual CA3 pyramidal cell layers in the *Dcx* KO differ from
7 each other and from WT, using LCM to separate *Dcx* KO layers at P0. Comparing WT to KO
8 layers revealed evidence of organelle, cell stress and metabolic gene differences. SPI and SPE
9 layer-specific gene changes were also identified, suggesting a difference in maturity between
10 the two layers at P0. Further comparisons with previously published datasets, as well as qPCR,
11 *in situ* hybridisation and BrdU labelling experiments, confirmed that many superficially-
12 positioned SPI cells have the identity of normally deeply-positioned, early-born neurons. Later-
13 born SPE cells had a more similar gene content and arrangement as WT cells. Thus, perturbed
14 birthdate-dependent organization in addition to separated localisations of SPI and SPE likely
15 lead to morphological and connectivity abnormalities (27).
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36 *Common transcriptome changes in SPI and SPE indicate abnormal organelles, increased cell*
37 *stress and metabolic abnormalities*
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41 The transcriptome experiment performed with laser microdissected material revealed
42 on average 45 % of the transcripts on the microarray showing a detectable level of expression,
43 falling into an appropriate range of the number of expressed genes expected per tissue, as shown
44 by previous RNAseq studies (67). Both KO layers showed appreciable numbers of differentially
45 expressed genes compared to WT, and these were for the majority layer-specific. This study of
46 isolated pyramidal cell populations in the hippocampus at P0 may reveal both cell-autonomous
47 and non-cell autonomous gene expression changes. A lack of *Dcx* has been shown previously
48 to result in abnormal microtubule bundling (68), as well as abnormal intracellular transport
49 (53), both intrinsic changes. However, exchanges with the environment (potentially also
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3 aberrant due to abnormal cell position) may be different from WT, resulting in either abnormal
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5 cellular responses, and / or abnormal effects on surrounding cells.
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8 Of the commonly deregulated genes in SPI and SPE compared to WT, many were
9
10 related to cellular response to stress, DNA damage repair pathways and organelles. DNA
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12 damage repair pathways, expressed highly during embryogenesis, were previously shown to be
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14 downregulated postnatally in several mouse lines (48). Two of the most significantly enriched
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16 functional categories in both KO layers were mitochondrion and Golgi apparatus (**Fig. 2, Supp.**
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18 **Table 5A**), corroborating our previous electron microscopy findings that showed ultrastructural
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20 abnormalities in these organelles in both SPI and SPE (30). Perhaps as a result of organelle
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22 dysfunction and/or increased cell stress, apoptotic cell death was also found to be increased in
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24 the hippocampus of *Dcx* KO mice at P2 (30).
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29 A direct link between *Dcx*, certain deregulated genes and organelle abnormalities seems
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31 plausible given the role of *Dcx* in microtubular functions (68) endocytosis (69), vesicular
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33 transport (49, 52, 53) and its interaction with molecular motors (49, 53). The misplacement or
34
35 impaired transport of particular organelles, and/or intracellular transport in general, due to
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37 abnormal microtubules (e.g. (68)) may affect metabolism, leading to cell stress and abnormal
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39 organelle morphologies. Furthermore, the function and subcellular localisation of mitochondria
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41 (70) and other organelles (71) play important roles in the formation of dendrites, which are
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43 abnormal in the *Dcx* KO (27). Converging data in the literature and the deregulated genes we
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45 identify hence point towards this being a potential mechanism leading to abnormal cell function.
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50 Linking metabolism and neuronal migration, oxidative phosphorylation was recently
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52 shown to be necessary for tangential migration of interneurons (72). Although CA3 neurons
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54 migrate radially, they travel further and over a longer period of time relative to CA1 (73), which
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56 could also make them more vulnerable to mitochondrial or metabolic abnormalities.
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Several metabolic genes are similarly deregulated in both KO layers compared to WT, which could also increase cell stress (**Table 2**). Increasing glycolysis in neurons (via genetic mechanisms) has been shown to increase cell stress (74). However, we cannot be certain that metabolic changes, particularly those related to glycolysis, are restricted to pyramidal neurons in our samples. We also found upregulation of genetic markers of interneurons (*Gad1*, *Sst*, *Dlx1*), oligodendrocyte precursor cells (OPCs) (e.g., *Olig1*) and astrocytes (e.g., *Glast*) in both SPI and SPE compared to WT. This may reflect disorganization of other cell types, and potentially non-cell-autonomous effects of *Dcx* mutation. For example, OPCs that do not express *Dcx* may migrate between the two pyramidal cell layers, being attracted to white matter that does not typically form there (75). Interneuron disorganization also seems apparent in the *Dcx* KO hippocampus, but this may be due to mislocalization of the pyramidal cells, invading spaces normally occupied by interneurons (*stratum radiatum* and *stratum oriens*). Alternatively, interneuron disorganization could also be a cell-autonomous effect as they normally express *Dcx*, and KO or knockdown causes slowed tangential migration (22, 23).

There are also several indicators of perturbed lipid metabolism in both SPI and SPE, which be linked to affect plasma membrane and organelle integrity, regardless of the cell types affected. This is interesting in light of previous links between lipid metabolism and epilepsy, and the efficacy of ketogenic diets in treating epilepsy in both mouse models and humans (76). There is however, no major evidence that *Dcx* KO mice experience epilepsy at P0 or *in utero*. Identified defects most likely contribute to the development of the heterotopia and epilepsy in adulthood. Thus, we can link various genetic and cellular defects potentially with epilepsy, and these associations to our knowledge have never previously been made for a heterotopia condition.

SPI contains more early-born, mature cells than SPE

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3 Differentially regulated genes related to migration, neuronal function and radial position
4 within the CA3 pyramidal cell layer suggest that SPI and SPE cells have different birthdates
5 and corresponding maturity statuses. Overall, the formation of two layers from E17.5 is most
6 probably related to a cell-autonomous lack of *Dcx*. Certain 'migration gene' changes, although
7 not all, are exclusive to either SPI or SPE. It is possible that the majority of early-born neurons,
8 which colonise CA3 and eventually become SPI, migrate normally and potentially in a *Dcx*-
9 independent fashion, whereas later-born neurons that primarily constitute SPE, do not migrate
10 far enough. It is however, difficult to assess whether SPE is correctly positioned compared to
11 WT, or if it indicates arrested migration in the intermediate zone. *In utero* genetic interventions
12 may address this point in the future.
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27 It seems unlikely that early-born neurons over-migrate given that *Dcx* mutations have
28 only been shown to decrease migration speed (77). SPI cell position close to the *stratum*
29 *radiatum* likely exposes them to an abnormal environment as they continue to mature, which
30 may further perturb patterns of gene expression. This could be one reason that the overlap in
31 previously published, early-born, outer-boundary gene lists was not more complete, although
32 the differences in age also likely contribute to this. Whether the abnormally positioned early-
33 born cells represent a specific subset of all early-born cells (e.g. (3, 5, 6) is currently unknown.
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43 Later-born neurons may be vulnerable to the loss of *Dcx*; they have farther to travel as
44 development proceeds, particularly in CA3 due to the curved shape. Furthermore, 'sojourning'
45 during migration occurs in this hippocampal field (2), and later-born mutant neurons may not
46 be able to reach earlier-born neurons after this pause. Radial glial cells may also have some
47 particularity in the CA3 region (7, 78). A decrease in migration speed may hence be revealed
48 more prominently in this region. As seen in migrating neuroblasts from the cortex (79) or in the
49 postnatal rostral migratory stream (RMS), *Dcx* knockdown slowed migration speed, and the
50 RMS neurons started to mature before reaching their normal location (80). The same situation,
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3 presumably for cell-autonomous reasons, could produce SPE in the lengthy trajectory of CA3,
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5 and these particular features of CA3 may also help explain why the entire hippocampus is not
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7 affected in the *Dcx* KO as it is in other mutants (*Lis1*, *Tuba1a*). Following this logic however,
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9 a complete inversion of all layers might be expected, which is not the case as revealed by the
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11 BrdU and *in situ* hybridisation data. Further studies are required to understand which
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13 subpopulations of early- and later-born neurons correctly or under-migrate and why. In the
14
15 future it will be interesting to compare embryonic expression patterns of e.g. up- or down-
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17 regulated SPE genes, which may reveal promising candidates for *in utero* genetic intervention.
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22 Similar disruption of hippocampal birthdate-dependent organization was also observed
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24 in the *reeler* mutant, even though the extent of disorganization was more severe (81). Also, our
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26 *in situ* hybridization experiments in the *Tuba1a* mutant were comparable to the *Dcx* KO,
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28 demonstrating perturbed inside-out lamination in CA3. This suggests overlapping mechanisms
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30 or increased vulnerability of particular neuronal populations depending on birthdate. It will be
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32 interesting in the future to also compare to *Lis1* mutants that have similar hippocampal
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34 phenotypes and to further focus on the differences between these models.
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39 To speculate further on this issue, we refer to the study by Prampero et al. (48), who
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41 analyzed gene transcription changes in whole brains from four lissencephaly mouse models
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43 (*Dcx*, *Lis1*, *Ndel1* and *14-3-3epsilon*) at three developmental time points. They observed that
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45 each mouse model had unique enrichment of particular gene clusters in addition to those that
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47 were commonly perturbed. We might expect similar findings if analogous hippocampal LCM
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49 transcriptome studies were performed in *Lis1* and *Tuba1a* mutant mice. However, these models
50
51 have more severe lamination defects that are present throughout the hippocampus, including
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53 two or more layers and fragmentation. *Lis1* mutants also exhibit variable neocortical defects
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55 depending on the gene dosage (17, 82). Unlike *Lis1*, where gene dosage appears critical for all
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57 migrating neurons, the decreased severity of the *Dcx* KO phenotype demonstrates that *Dcx* is
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3 not essential for most of the mouse hippocampus and the neocortex to develop normally, and
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5 that the CA3 later-born (SPE) cells are perhaps the most vulnerable to *Dcx* mutation. Our gene
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7 expression data in different cell populations for various cellular processes (e.g. upregulation of
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9 cell adhesion in SPI versus WT, and deregulated morphology and cytoskeleton gene clusters in
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11 SPI or SPE versus WT) show concordance with the Pramparo et al. study, however, with the
12
13 caveat that differences are also expected when comparing whole brain to microdissected
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15 hippocampus. It remains to be seen which other cellular processes may be uniquely deregulated
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18 in *Dcx* KO mice compared to the other models.
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24 *Dcx* KO network formation

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26 The disorganization of pyramidal cells and/or interneurons in the *Dcx* KO may impair network
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28 formation and lead to epileptogenesis. A subset of early-born GABAergic neurons function as
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30 hubs, generating giant depolarizing potentials (GDPs) that synchronize neural firing across the
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32 postnatal hippocampus *in vitro* (83, 84). Potential interneuron disorganization associated with
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34 both SPI and SPE (as suggested by the transcriptome data) might change their function, or have
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36 an effect on developing networks.
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41 Pyramidal cells with the same birthdate show selective interconnectivity in the
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43 trisynaptic circuit (5), with some early-born CA3a/b pyramidal neurons functioning as highly
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45 interconnected hub neurons in juvenile mice *in vitro* (6). With a large number of early-born
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47 neurons being misplaced into a distinct layer (SPI), it is likely that these normal developmental
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49 patterns are perturbed. Another important aspect of circuit formation depends on the clonal
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51 relationship of sister pyramidal cells, which share interneuron connectivity (7). We do not know
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53 if neurons derived from the same progenitor cell distribute across SPI and SPE, or whether they
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55 tend to group together into one of these layers, but either formation could be pathological in
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57 terms of network formation. The time of onset of abnormal electrical activity (or seizures) is
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3 currently unknown in this model, but given the finding that early-born neurons are abnormally
4 positioned and considering the important role of birthdate-dependent network synchronization
5 outlined in the studies above, it seems likely that postnatal neuronal activity will be
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10 subsequently perturbed.
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12 13 14 15 *Conclusion*

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17 We show here an advanced molecular analysis of the heterotopic CA3 layers in *Dcx* KO
18 mice which reveals organelle, cell stress and metabolism abnormalities as well as abnormal
19 positioning of SPI and SPE. It remains to be seen what repercussions the relative displacement
20 of early-born, outer boundary cells might have on neuronal differentiation, cell-to-cell
21 communication, connectivity and function. It remains possible that SPI cells, exhibiting a
22 unique molecular identity, still become connected appropriately, even though they are separated
23 from the environment of SPE cells. The mechanisms leading to misplaced early-born versus
24 late-born neurons still remain unknown, but may be related to different migration mechanisms,
25 especially in the complex CA3 region (85). The abnormal neuronal position for rostral-dorsal
26 SPE cells, lacking an outer-boundary with the *stratum oriens*, could also have consequences on
27 connectivity. Further studies are required, including investigating synaptogenesis during
28 postnatal stages, and performing gene expression analyses of more mature *Dcx* KO cells, to
29 help answer these questions concerning the consequences of abnormal neuronal position on
30 neuronal and network function.
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MATERIALS AND METHODS

Animals

Dcx KO and WT mice were maintained on the *Sv129Pas* background with more than ten generations of backcrosses (19, 22). Genotyping was performed by PCR to verify the inactivation of the *Dcx* gene in KO animals (22). All experiments were performed in accordance with institutional, national and international guidelines (EC directive 86/609) and were approved by the local ethical committees (French MESR N°: 00984_02). The day of confirmation of vaginal plug was defined as embryonic day zero (E0.5), and the day of birth was defined as postnatal day zero (P0). We considered this latter timepoint to be suitably late to reduce the study of mixed populations of migrating and differentiating neurons, in favour of studying just differentiating neurons, and suitably early to be before epileptic seizures begin. P0 animals were anesthetized by placing on ice for 5 minutes before decapitation. For laser capture microdissection (LCM), the whole head was immediately frozen in isopentane at -35°C for 1 minute before storing at -80°C for future cryostat cutting. For *in situ* hybridization and immunohistochemistry experiments, animals were perfused, and brains were fixed using 4% paraformaldehyde (PFA) in phosphate buffered saline (PBS). Brains were post-fixed in the same solution overnight. Embryonic and early postnatal brains were cryoprotected in 30% sucrose, embedded and frozen in OCT tissue freezing medium, and cut using a cryostat (Leica) at 20 µm thickness, and spread on SuperFrost II slides.

Laser capture microdissection (LCM)

Coronal brain sections (12 µm) containing the rostro-caudal hippocampus were prepared using a cryostat (Leica) maintained at -20°C and mounted on PENmembrane slides (1440–1000, PALM, Bernried, Germany) which were pre-treated by RNase ZAP (Ambion) and UV irradiated in a cell culture hood for 30 min at 254 nm. After sectioning, the slides were stored

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3 in a -80°C freezer for use within 1 week. On the day of LCM, the slides were removed from the
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5 freezer, and fixed in 70% ethanol, prepared in RNase free water for 2 min and then in 50%
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7 ethanol for 5 seconds, and stained with 1% cresyl violet (Sigma) for 1 min. Subsequently, slides
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9 were rinsed and dehydrated using serial dilutions of ethanol (50% for 5 sec, 75% for 5 sec,
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11 100% for 30 sec). Sections were air-dried and subjected to LCM within the next 30 minutes.
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13 Samples were cut using a Zeiss LCM system (PALM Microbeam). In WT animals, the
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15 hippocampal CA3 region was microdissected in one piece. In *Dcx* KO animals, internal *stratum*
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17 *pyramidale* (SPI) and external *stratum pyramidale* (SPE) CA3 cells in the two layers were
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19 separated. Per brain, the entire CA3 structure (bilateral) from the dorsal hippocampus was
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21 excised; for each condition 60 sections per animal (n=5) were pooled.
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28 *RNA isolation, quantity and quality assessment*

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30 Total RNA was isolated from pooled microdissected material using an RNA isolation kit
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32 (Arcturus picopure) according to manufacturer instructions. RNA for each brain was eluted in
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34 13 µl of the elution buffer provided in the kit. RNA quantity was measured using a nanodrop
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36 spectrophotometer. RNA quality was checked using an Agilent 2100 Bioanalyzer with the RNA
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38 6000 Pico LabChip Kit (5065-4473, Agilent Technologies, Palo Alto) according to the
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40 manufacturer's instructions (**Supp Fig 2**). No statistical differences in RNA qualities ('RIN'
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42 values) were seen between the samples.
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48 *Linear RNA amplification and GeneChip hybridization*

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50 15 samples, corresponding to n=5 samples for each condition were processed using the Illumina
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52 TotalPrep RNA Amplification Kit (Ambion, Life Technologies) and the Whole-genome Gene
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54 Expression Direct Hybridization Assay (Illumina) according to manufacturers' instructions.
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56 Briefly, 150-200 ng total RNA was used to prepare double-stranded cDNA using a T7 oligo
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58 (dT) primer (Illumina protocol). Reverse transcription was followed by *in vitro* transcription in
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3 the presence of biotinylated nucleotides. cRNA samples were hybridized to the Illumina
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5 Mouse-Ref-8 expression beadchip arrays in the appropriate buffer overnight at 58 °C. After
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7 hybridization and washes, fluorescent tagging was achieved by incubation with streptavidin-
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9 Cy3. Each array contains approximately 25,600 well-annotated RefSeq transcripts, represented
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11 as oligonucleotides attached to beads (average of 30 beads/per transcript), corresponding to
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13 over 19,100 unique genes.
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16 17 18 *Data analysis*

19 20 21 *Intra-sample normalisation*

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23 In order to compare gene expression intensities between samples, expression values within
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25 samples were first normalised to account e.g. for differences in mRNA library preparation. To
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27 this end, each log-transformed expression value was corrected by subtracting the median of the
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29 distribution of log-transformed expression values and dividing by the standard deviation of this
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31 distribution of log-transformed expression values and dividing by the standard deviation of this
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33 distribution (**Supp Figure 3A**).
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35 36 37 *Inter-sample normalisation*

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39 Samples were distributed on two different slides, requiring a correction for potential
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41 experimental or technical differences between slides. A principal component analysis of the
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43 expression values of the 15 samples (**Supp Figure 3B, C**) showed that the principal eigenvector
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45 ($Z[1]$) can separate the samples according to their slide of origin. To normalize, we averaged
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47 the expression values of each gene across the four SPI and the four SPE samples of slide 1
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49 respectively. We subtracted this average value from the value of the single SPI and SPE samples
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51 on slide 2 (respectively Δ_I and Δ_E in **Supp Figure 3D**). When data points are close to zero on
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53 both axes, this indicates that slides 1 and 2 show the same expression values. While this appears
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55 to be true for many genes, we also observed a distribution of points along a slope. For genes
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57 lying on this diagonal, this indicates a bias of the same magnitude for both SPI and SPE samples.
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We first performed a gene-wise correction of the SPI samples on slide 1 to be compatible with corresponding samples on slide 2. The corrected value for each gene j is obtained as the sum of the original value and an estimate of the bias error. The estimate of the bias is based on the bias of SPE (since we assume this to be independent of the sample, i.e. affecting intensities in the same way on the two slides) weighted with a constant w_I .

The correction for SPI samples is:

$$I_j^{corr,slide_1} = I_j^{slide_1} + w_I \cdot \Delta_{E,E,j}$$

$$\text{with: } \Delta_{E,j} = \text{average}(E_j^{slide_1}) - E_j^{slide_2}$$

where I and E represent the intensity of gene j on the slide indicated by the superscript (the subscript of the sample on the slide is omitted for better readability). The constant w_I was determined by minimizing the error between the intensities of the genes on two different slides according to a least square criteria (as in the equations below).

$$\text{For SPI: } \min_{w_I} \left[\sum_j \left(I_j^{slide_2} - \text{average}(I_j^{corr,slide_1}) \right)^2 \right]$$

$$\text{For SPE: } \min_{w_E} \left[\sum_j \left(E_j^{slide_2} - \text{average}(E_j^{corr,slide_1}) \right)^2 \right]$$

Similarly, the SPE samples were corrected on slide 1 with the bias estimated from SPI and thus all samples were aligned to the samples as found on slide 2.

After corrections, principal component analysis showed that SPI and SPE samples from slide 2 respectively cluster with the SPI and SPE samples from slide 1.

Finally the linear model fit of the LIMMA package (86) was applied on these bias-corrected expression values in order to detect the differentially expressed genes pertaining to the three conditions, using a t -test. The Benjamini-Hochberg false discovery rate control (with $P = 0.05$) was applied to correct for multiple testing. Results are presented as ratios between the conditions.

Pathway analysis and functional category clustering

A global overview of the biological processes and enriched functional clusters in SPI and SPE lists was obtained using a Database for Visualization and Integrative Discovery (DAVID) (Dennis et al., 2003). The following sources were used: COG-Ontology, SP-PIR-Keywords, UP-SEQ-Feature, GOterm-BP-FAT, GOterm-CC-FAT GOterm-MF-FAT, KEGG pathways, and INTERPRO protein information resources. Analysis was performed using the default settings (medium stringency); enrichment scores > 1.5 were considered significant.

For further specific questions, Ingenuity Systems Pathway Analysis (IPA) (Ingenuity Systems, Mountain View, CA; www.ingenuity.com) was used to define enriched functional categories and pathways specific to the developing central nervous system.

Quantitative PCR (qPCR) validation

Linear amplified LCM RNAs (Ambion Life Technologies or Nugen Ovation kit) were used to prepare cDNA. Real time qPCR assays using the SYBRgreen method followed MIQE guidelines (87). Gene-specific primers were designed using Primer Express Software (PE Applied Biosystems, **Supp Table 8**), except for *Atp1b1*, which were purchased from Sigma (KSPQ12012, primer set 1). Standard curves were generated from assays made with serial dilutions of cDNA to calculate PCR efficiencies ($90\% < \text{efficiency} < 105\%$, with $r^2 \geq 0.998$). Threshold cycles (C_t) were transformed into quantity values using the formula $(1 + \text{Efficiency})^{-C_t}$. Only means of triplicates with a coefficient of variation of less than 10 % were analysed. Inter-plate variation was below 10 %. Values were normalized to the geometric mean of 3 Normalization Factors (NF) found to be the most stable through all samples using the geNorm approach. These were ATP synthase, H⁺ transporting mitochondrial F1 complex, beta subunit (*Atp5b*), eukaryotic translation initiation factor 4A2 (*Eif4a2*) and prosaposin (*Psap*). Average

1
2
3 values \pm standard deviations were compared for 3-5 animals of each genotype. Ratios were
4
5 calculated and *P* values using the Student *t*-test.
6
7

8 9 *mRNA in situ hybridization*

10
11 Specific antisense RNA probes (see **Supp Table 8**) for *Col6a1*, *Necab2*, *St18* and *Grp* genes
12
13 were used for *in situ* hybridization analyses. Equivalent sense probes were also generated for
14
15 comparison. Digoxigenin (DIG) probes were synthesized with a labeling kit according to the
16
17 manufacturer's instructions (Roche Diagnostics). Following a protocol adapted from Bally-Cuif
18
19 and Wassef (1994) (88), frozen cryostat sections were rinsed 3 \times 5 min in PBS, postfixed in 4%
20
21 PFA, rinsed in PBS 2 \times 5 min, and treated with proteinase K (10 μ g/ml) for 10 min. Sections
22
23 were incubated in PBS + glycine (2 mg/ml), rinsed in PBS, and postfixed in a mixture of 4%
24
25 PFA, followed by a rinse in PBS. Tissue sections were hybridized at 70°C overnight with the
26
27 DIG-labeled probes diluted 1/100 in hybridization buffer (50% deionised formamide, 10%
28
29 dextran sulphate, 1 mg/ml Yeast RNA, 1x Denhardt's solution). The next day, sections were
30
31 sequentially washed in 2X saline sodium citrate (SSC) Tween 0.1% at 70°C then in maleate
32
33 buffer (Maleic acid 100 mM, NaCl 150 mM, 0.1% Tween20, pH 7.5) at room temperature. For
34
35 immunological detection of DIG-labeled hybrids, sections were first blocked (2% blocking
36
37 reagent-Roche Applied Science, cat. 1096176, 20% sheep serum in maleate buffer) and then
38
39 incubated overnight at 4°C in the same solution containing sheep anti-DIG-alkaline
40
41 phosphatase-conjugated Fab fragments (Roche Diagnostics) diluted 1/2000. The following day,
42
43 sections were washed 4 \times 15 min in maleate buffer and 30 min in NTMT buffer (100 mm NaCl,
44
45 100 mm Tris-HCl, pH 9.5, 50 mm MgCl₂, 0.1% Tween 20). The alkaline phosphatase
46
47 chromogen reaction was performed in NTMT buffer containing 100 mg/ml nitroblue
48
49 tetrazolium (Roche Diagnostics) and 50 mg/ml 5-bromo-4-chloro-3-indolyl phosphate (Roche)
50
51 at room temperature for 1-7 days and stopped with PBS. Sections were mounted on glass slides,
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1
2
3 dried, counter-colored using nuclear fast red and dehydrated in graded ethanol solutions, and
4
5 coverslipped with Vectamount (Vector Laboratories).
6
7

8 9 *Birthdate studies*

10
11 Pregnant mice were injected intraperitoneally (i.p) four times with 2 hour intervals at E12.5
12
13 with 100 mg/kg bromo deoxy uridine (BrdU), or once with 200 mg/kg BrdU at E16.5. Animals
14
15 were sacrificed at P21-24. To determine the number of BrdU-positive cells, every tenth section
16
17 of 20 μ m (200 μ m intervals) of the dorsal hippocampus from both cerebral hemispheres was
18
19 processed for immunohistochemistry (n = 3-5 animals per genotype per BrdU injection date).
20
21
22
23
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25
26

27 *Immunohistochemistry*

28
29
30 Cryostat sections at 20 μ m thickness were washed in PBS 1X, and incubated in 2 N HCl at
31
32 37°C for 40 min to denature DNA. Sections were thoroughly washed in PBS 1X to neutralize
33
34 the acid, and incubated in blocking solution (0.1 M PBS, 0.3% Triton X-100, 2% normal goat
35
36 serum) for two hours at room temperature. This was followed by incubation with the primary
37
38 antibodies; anti-BrdU (Rat, clone ICR1, ABD serotec 1:100), anti-NeuN (Mouse, clone A60
39
40 Millipore, 1:200); diluted in blocking solution, overnight at 4°C. Brain sections were then
41
42 washed three times in PBS 1X and incubated with fluorochrome-conjugated secondary
43
44 antibodies (1:400, Invitrogen) diluted in blocking solution containing Hoechst fluorescent
45
46 nuclear counterstain (1:5000). Sections were mounted using fluoromount G (Electron
47
48 Microscopy Devices) and cover-slipped.
49
50
51
52

53 54 *Image acquisition and quantification*

1
2
3 Bright-field *in situ* hybridization images were acquired with a Coolsnap CCD camera fitted to
4
5 a Provis Olympus Microscope using 4X, 10X and 20X objectives (magnification/numerical
6
7 aperture).
8
9

10
11 Fluorescent BrdU and NeuN immunostained sections were acquired using SP5II Leica
12
13 confocal microscope using a 40X objective. BrdU-labelled cell quantification was performed
14
15 using Icy software (Version 1.5.4.2) and the Spot Detector module (89). BrdU-labelled cells
16
17 were counted using the following criteria: 1. Threshold parameters were optimised for inclusion
18
19 of only strongly labelled BrdU cells, and identical settings were applied for counting of all
20
21 images. 2. Co-immunostaining for both BrdU and NeuN was required to exclude astrocytes.
22
23 Division of the hippocampal CA3 subfields in coronal brain sections into CA3a, CA3b and
24
25 CA3c was performed as described previously (90). The WT pyramidal cell layer was divided
26
27 in half on the radial axis, described as “inner” and “outer”, for comparison to the KO bilayers.
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34 **Acknowledgements**

35
36 We thank Nicolas Narboux-Neme, Sylvie Thomasseau, Katia Boutourlinsky, Richard
37
38 Belvindrah and Gael Grannec for technical help and advice, Evelyne Souil for initial advice
39
40 concerning LCM, David Gentien for advice and use of the Institut Curie platform for Picochip
41
42 analyses and Dominique Wendum for the use of the Saint Antoine LCM platform. We thank
43
44 the Inserm Avenir program, the French Agence National de la Recherche (ANR- 08-MNP-013),
45
46 the European Union's Seventh Framework Programme for research, technological development
47
48 and demonstration under grant agreement no: Health-F2-602531-2013, the CNRS, the
49
50 Fondation Bettencourt Schueller and the Fondation Jérôme Lejeune for grant support to FF. We
51
52 thank University Paris 6 and An-Najah University, Palestine for support to RKN. We thank the
53
54 IFM imaging and animal house facilities. We thank the Ile de France region for support of
55
56 imaging and animal house facilities and the CNRS TAAM for mouse housing. MS was funded
57
58
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1
2
3 by the European project DESIRE. FF and MS are associated with the BioPsy Labex project and
4
5 the Ecole des Neurosciences de Paris Ile-de-France network.
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10 **Conflict of Interest Statement.** None declared.
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For Peer Review

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FIGURE LEGENDS

Figure 1

WT versus *Dcx* KO hippocampus at late embryonic and early postnatal stages. (A, B) Cresyl violet stained coronal sections of the hippocampus at E18.5 and P0, prior to laser capture microdissection cutting. The CA1 and CA3 fields of the pyramidal cell layer are indicated on the WT section. Arrows on the *Dcx* KO section indicate the double pyramidal cell layer. So, *stratum oriens*, sr, *stratum radiatum*. (C) Laser capture microdissection from cresyl violet stained P0 brain sections. Hippocampal CA3 WT and *Dcx* KO internal (SPI) and external (SPE) layers are shown, with schematized indications of areas selected for excision. Scale bars, 300 μm E18.5; 300 μm (B) and 70 μm (C) P0.

Figure 2

Global gene expression changes in the two *Dcx* KO layers, compared to WT. (A) Increasing numbers of differentially expressed genes as *P* value cutoffs are lowered. SPE versus WT, SPI versus WT, and SPI versus SPE comparisons are shown. The y-axis indicates numbers of genes. The red color indicates up-regulated, and the green color indicates down-regulated genes. (B) Genes commonly de-regulated in SPI and SPE were subjected to DAVID functional clustering analyses. Each significant functional category (y-axis), is plotted against the value of enrichment score (x-axis) obtained using the DAVID pathway analysis software. DAVID analyses are also resumed in Supp Table 5.

Figure 3

DAVID functional clustering of SPE and SPI layer-specific gene expression differences (A-D). Functional clustering of up-regulated and down-regulated differentially expressed genes from comparisons of SPI cells to WT (A,B), and SPE cells to WT (C,D), respectively. Each

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3 significant functional category (y-axis), is plotted against the value of enrichment score (x-
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5 axis).
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10 **Figure 4**

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12 Validation of transcriptome data with quantitative RT-PCR (qPCR) and *in situ* hybridizations
13 (ISH). (A) qPCR analyses for selected genes with comparison to transcriptome data. Y-axis
14 corresponds to relative fluorescence units. Up-regulation compared to WT is indicated as
15 positive values and down-regulation as negative ($P < 0.05$, Student's *t*-tests). Results are
16 presented as the ratio of means \pm SEM ($n = 3-5$ samples per genotype). (B,C) ISH in coronal
17 sections showing layer-specific CA3 gene expression patterns. In the *Dcx* KO CA3 at P0 (B),
18 *Necab2* and *St18* are primarily expressed in SPI, whereas *Grp* is primarily expressed in SPE,
19 as well as in late-migrating neurons in the intermediate zone (IZ) in both the KO and WT. (C)
20 ISH in coronal sections (R, rostral, C, caudal) in adults. The WT section is equivalent to the
21 most rostral KO section. *Col6A1* and *St18* label outer-boundary cells closest to the *stratum*
22 *oriens* in WT, whereas mainly SPI cells are labeled in the *Dcx* KO in septal regions, as well as
23 some outer boundary cells in more temporal regions. *Necab2* labels CA2 cells and CA3 outer-
24 boundary cells in WT, and similar to *Col6A1* and *St18*, shows a predominant expression in SPI
25 cells in septal regions.
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48 **Figure 5**

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50 BrdU labeling of early- and late-born neurons. Representative immunohistochemistry images
51 of dorsal hippocampi from WT (left) and *Dcx* KO males exposed to BrdU at E12.5 (A) and
52 E16.5 (B) show BrdU (green) and NeuN (red) labeling. White arrow heads indicate co-labeled
53 cells. Bar graphs depict the average percent (\pm SEM) of total BrdU+ neurons (NeuN+) found
54 in SPI (inner) and SPE (outer) heterotopic layers of *Dcx* KO mice, or the inner and outer halves
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3 of the single WT pyramidal layer. The proportions of BrdU+ neurons located in the inner and
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5 outer layers, born at either E12.5 or E16.5, were significantly different from WT in the CA3
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7 region and each subregion (CA3a, b, c; Chi-squared tests, $p < 0.05$, $n = 4-5$ mice per genotype
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9 per injection time). Significant differences between the percentages of total BrdU+ neurons
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11 located in SPI/inner and SPE/outer are indicated (paired t -tests, $*p < 0.05$, $**p < 0.01$, $***p <$
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13 0.001).
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For Peer Review

Table 1. Stress-response genes significantly ($P < 0.01$) changed in SPI and/or SPE vs. WT. Values are ratios. Values in parentheses, $P < 0.05$.

	SPI vs. WT	SPE vs. WT
DNA stress response		
High mobility group box 2 (Hmgb2)	1,332	1,185
Structural maintenance of chromosomes 6 (Smc6)	1,202	1,209
Transformation related protein 53 binding protein 1 (Trp53bp1)	1,195	1,320
Excision repair cross-complementing rodent repair deficiency, complementation group 5 (Ercc5)	1,157	1,202
FGF receptor activating protein 1 (Frag1)	1,155	1,191
Myeloid differentiation primary response gene 116 (Myd116)	1,133	1,074
Structural maintenance of chromosomes 3 (Smc3)	1,100	
Polynucleotide kinase 3'-phosphatase (Pnkp)	0,913	0,874
Serum/glucocorticoid regulated kinase 1 (Sgk1)	0,875	0,900
Structural maintenance of chromosomes 1A (Smc1a)	0,772	0,907
Antioxidants		
Glutathione S-transferase, mu 1 (Gstm1)	1,287	1,181
Glutathione S-transferase, mu 5 (Gstm5)	1,259	
Sulfiredoxin 1 homolog (<i>S. cerevisiae</i>) (Srxn1)	1,145	
Chaperones/Heat shock proteins		
Heat shock protein 90kDa alpha (cytosolic), class B member 1 (Hsp90ab1)	1,281	1,553
Heat shock protein 1 (chaperonin) (Hspd1)	1,271	1,235
DnaJ (Hsp40) homolog, subfamily A, member 2 (Dnaja2)	1,162	1,165
Heat shock protein 2 (Hspa2), transcript variant 1	1,159	
Heat shock protein 1 (Hspb1)		0,874
Homocysteine-inducible, endoplasmic reticulum stress-inducible, ubiquitin-like domain member 1 (Herpud1)	0,894	0,906
Hspb associated protein 1 (Hspbap1)	0,872	
Heat shock protein 8 (Hspa8)	0,773	
Other		
Pleiotrophin (Ptn)	1,941	
Metallothionein 1 (Mt1)	1,814	
Metallothionein 3 (Mt3)	1,646	(0,784)
Aldehyde dehydrogenase 1 family, member L1 (Aldh1l1)	1,331	1,123
Aldehyde dehydrogenase family 3, subfamily A2 (Aldh3a2)	0,891	
Aldehyde dehydrogenase 4 family, member A1 (Aldh4a1)	0,819	
Aldehyde dehydrogenase 1 family, member L2 (Aldh1l2)	0,774	
Solute carrier family 6 (neurotransmitter transporter, L-proline), member 7 (Slc6a7)	1,544	0,821
Metabolic stress response		
Aldolase C, fructose-bisphosphate (Aldoc)	2,116	
Enolase 2, gamma neuronal (Eno2)	1,281	

Table 2. Metabolic genes significantly deregulated in KO layers ($P < 0.01$). Values are ratios. Values in parentheses, $P < 0.05$ **A. Genes up- or downregulated in SPI and SPE vs. WT**

Definition	SPI vs. WT	SPE vs. WT
Aldolase C, fructose-bisphosphate (Aldoc)	2,116	1,326
Solute carrier family 1 (glial high affinity glutamate transporter), member 3 (Slc1a3); Glast	1,638	(1,199)
Phosphofruktokinase, platelet (Pfkp)	1,639	1,288
Diazepam binding inhibitor (Dbi), transcript variant 2; Acap	1,509	1,163
Isocitrate dehydrogenase 3 (NAD ⁺), gamma (Idh3g)	1,148	(1,124)
ATP synthase, H ⁺ transporting, mitochondrial F1 complex, alpha subunit, isoform 1 (Atp5a1)	1,282	1,272
Enolase 2, gamma neuronal (Eno2)	1,281	0,838
Acyl-CoA thioesterase 1 (Acot1)	1,280	1,213
Sphingomyelin phosphodiesterase, acid-like 3A (Smpd13a)	1,269	1,162
Cytochrome c oxidase subunit VIIa polypeptide 2-like (Cox7a2l)	1,254	(1,091)
Cytochrome C oxidase assembly factor 5 (Coa5)	1,210	1,160
Phosphoglycerate mutase 1 (Pgam1)	1,192	1,128
Pyruvate dehydrogenase (lipoamide) beta (Pdhb)	1,172	1,269
Sphingomyelin phosphodiesterase 1, acid lysosomal (Smpd1)	1,156	1,170
Hexokinase 1 (Hk1)	(1,108)	1,214
Sphingomyelin phosphodiesterase 4 (Smpd4)	(1,068)	1,120
Acetoacetyl-CoA synthetase (Aacs)	0,906	0,907
Phosphatidylglycerophosphate synthase 1 (Pgs1)	0,867	0,827
Mitochondrial Translocator Assembly And Maintenance Protein, Homolog (<i>S. Cerevisiae</i>) (Tam41)	0,836	1,125
Nitric oxide associated 1 (Noa1)	0,834	0,894
ATP citrate lyase (Acly)	0,831	0,867
Hydroxysteroid (17-beta) dehydrogenase 7 (Hsd17b7)	0,823	0,862
Transaldolase 1 (Taldo1)	0,806	0,847
Plasticity-related protein 3/phospholipid phosphatase related 1 (Plppr1)	0,796	(1,177)
Mevalonate (diphospho) decarboxylase (Mvd)	0,780	0,759

B. Genes up- or downregulated in SPI vs. WT

Definition	SPI vs. WT	SPE vs. WT
Aldehyde dehydrogenase 1 family, member L1 (Aldh1l1)	1,391	
Aldolase A, fructose-bisphosphate (Aldoa)	1,360	
Enolase 1, alpha non-neuron (Eno1)	1,275	
Solute carrier family 2 (facilitated glucose transporter), member 1 (Slc2a1); Glut1	1,190	
Malate dehydrogenase 2, NAD (mitochondrial) (Mdh2)	1,160	
Solute carrier family 2 (facilitated glucose transporter), member 3 (Slc2a3); Glut3	1,113	
6-phosphofructo-2-kinase/fructose-2,6-biphosphatase 2 (Pfkfb2)	0,877	
6-phosphofructo-2-kinase/fructose-2,6-biphosphatase 4 (Pfkfb4)	0,835	

Table 3. Neurodevelopmental genes significantly deregulated in KO layers ($P < 0.01$).
 Genes highlighted in dark gray and light gray were upregulated and downregulated, respectively, in both SPI and SPE vs. WT.

SPI vs WT				SPE vs WT			
Upregulated		Downregulated		Upregulated		Downregulated	
Gene	Ratio	Gene	Ratio	Gene	Ratio	Gene	Ratio
Ptn	1,941	Dcx	0,521	Ppp1cb	1,408	Dcx	0,577
Rasgrp1	1,825	Rnd2	0,625	Dlx1	1,372	Rasgrp1	0,771
Slc1a3	1,638	Tiam2	0,669	Capn2	1,282	Ppp1r1a	0,872
Pdgfra	1,540	Efnb1	0,781	Rasgrf1	1,271	Ppp1ca	0,885
Wnt5a	1,522	St8sia2	0,802	Neurod1	1,245	Gas6	0,888
Gad1	1,490	Nr2f2	0,809	Nckap1	1,229	Ppp2ca	0,888
Ppp1r3c	1,489	Cdk5	0,841	Ppp2r2b	1,227	Ap2a2	0,896
Reln	1,487	Ap1m1	0,851	Gad1	1,225	Robo1	0,899
Dlx1	1,482	Mark2	0,852	Ncam1*	1,224	Dcl1	0,899
Il16	1,480	Katna1	0,856	Sst	1,216	Gdnf	0,913
Mapt*	1,443	Srf	0,859	Ctnnb1*	1,207	Pou4f1	0,944
Ccnd1	1,316	Lmn2	0,865	Lmn1	1,196		
Ppp2r2c	1,309	Ptk2	0,874	Tpm3	1,195		
Rasgrf1	1,309	Ap2m1	0,897	Pafah1b1*	1,174		
Ctnnb1*	1,301	Pak4	0,907	Ppp2r1b	1,171		
Enpp2	1,290	Itgb1bp1	0,910	Rhob	1,170		
Robo1	1,266	Prkcz*	0,914	Tubgcp2	1,166		
Sst	1,264	Pou4f1	0,947	Dab1	1,144		
Rhob	1,251			Prkcz*	1,103		
Ncam1*	1,235						
Nckap1	1,217						
Prkaca	1,215						
Cav1	1,214						
Kif2a	1,206						
Ppp1r14c	1,205						
Cx3cl1	1,200						
Flt1	1,195						
Nde1*	1,194						
Kif1a	1,192						
Stat3*	1,189						
Ednrb	1,185						
Unc5c	1,170						
Nfasc	1,155						
Nos3	1,153						
Gas6	1,150						
Ap2a2	1,149						
Map2k4	1,143						
Dab1	1,132						
Cdkn2c	1,127						
Angpt2	1,121						
Chi3l1	1,117						
Pdgfrb	1,108						

*Probe dependent

Table 4. Genes related to epilepsy, excitability, and/or neurotransmission upregulated in SPI vs. WT ($P < 0.01$). Values are fold changes. Values in parentheses, $P < 0.05$

Definition	SPI vs. WT
ATPase, Na ⁺ /K ⁺ transporting, alpha 2 polypeptide (Atp1a2)	1,657
ATPase, Na ⁺ /K ⁺ transporting, beta 1 polypeptide (Atp1b1)	1,283
ATPase, Na ⁺ /K ⁺ transporting, beta 2 polypeptide (Atp1b2)	1,350
Leucine-rich repeat LGI family, member 1 (Lgi1)	1,313
Synaptic vesicle glycoprotein 2 a (Sv2a)	1,218
Potassium large conductance calcium-activated channel, subfamily M, beta member 4 (Kcnmb4)	1,213
Potassium voltage-gated channel, shaker-related subfamily, member 1 (Kcna1)	1,157
Potassium voltage-gated channel, shaker-related subfamily, member 2 (Kcna2)	1,125

Table 5. SPI gene expression compared to outer-boundary and early-born CA3 transcriptome studies ($P < 0.01$). Values are ratios. Genes common in A and B are underlined.

A. Genes commonly up- or down-regulated in outer-boundary pyramidal neurons of adult CA3 and SPI (Thompson et al., 2008; Table S2)

Upregulated	SPI vs. WT	Upregulated	SPI vs. SPE
Necab2	1,636	Necab2	1,697
<u>St18</u>	1,634	<u>St18</u>	1,641
Lypd1	1,405	Fxyd5	1,639
Fxyd5	1,367	Fxyd7	1,639
Fxyd7	1,367	Sema5a	1,471
Epha7	1,344	Lypd1	1,417
Sema5a	1,330	<u>Stard13</u>	1,226
Cdh13	1,222	Cdh13	1,211
<u>Stard13</u>	1,220	Slc30a3	1,112
Kcnp3	1,155	Klotho	1,103
Thrsp	1,143	Downregulated	SPI vs. SPE
Kcnf1	1,105	Efnb3	0,922
Klotho	1,088	Cadps2	0,924

B. Genes upregulated in *Lsi1* and/or *Lsi2* pyramidal neurons of adult CA3 compared to SPI (Deguchi et al., 2011; Supp. Fig. 3)

Upregulated	SPI vs. WT	Upregulated	SPI vs. SPE
<u>St18</u>	1,634	<u>St18</u>	1,641
Rfx4	1,260	Slc40a1	1,313
Slc40a1	1,242	<u>Stard13</u>	1,226
<u>Stard13</u>	1,220	Rfx4	1,120
Dleu7	1,139		
Wwtr1	1,116		
Downregulated		Downregulated	
Fzd7	0,687	Fzd7	0,633
D16Ert472e	0,756	Odz4	0,684
Odz4	0,820	D16Ert472e	0,787
Ptpd	0,911		

Genes downregulated in *Lsi1* and/or *Lsi2* pyramidal neurons of adult CA3 compared to SPI (Deguchi et al., 2011; Supp Fig 3)

Upregulated	SPI vs. WT	Upregulated	SPI vs. SPE
Hba-a1	1,874	Lmna	1,240
Epha5	1,226	Gabbr3	1,158
Haghl	1,193	Lrrc33	1,128
Prss35	1,153	Ube3a	1,069
Dlgap2	1,110		
Lrrc33	1,089		
Downregulated		Downregulated	
Zfp277	0,821	Mid1	0,762
Csnk1e	0,885	Use1	0,825
Ap2m1	0,897	Zfp277	0,853
Entpd5	0,906		
Creb3	0,932		
Rad50	0,939		

ABBREVIATIONS

SPI- *stratum pyramidale* internal

WT- wildtype

KO- knockout

SPE- *stratum pyramidale* external

RT-PCR- Reverse transcriptase PCR

qPCR- quantitative RT-PCR

ISH- *in situ* hybridisation

LCM- laser capture microdissection

RIN- RNA integrity number

DAVID- Database for Annotation, Visualization and Integrated Discovery

IPA- Ingenuity Systems Pathway Analysis

P0- postnatal day 0

BrdU- bromodeoxyuridine

MAP- microtubule associated protein

EEG- electroencephalogram

SBH- subcortical band heterotopia

Dcx- doublecortin

ID- intellectual disability

ROS- reactive oxygen species

HSP- heat shock protein

NADH (NADPH)- Nicotinamide adenine dinucleotide (phosphate)

CNS- central nervous system

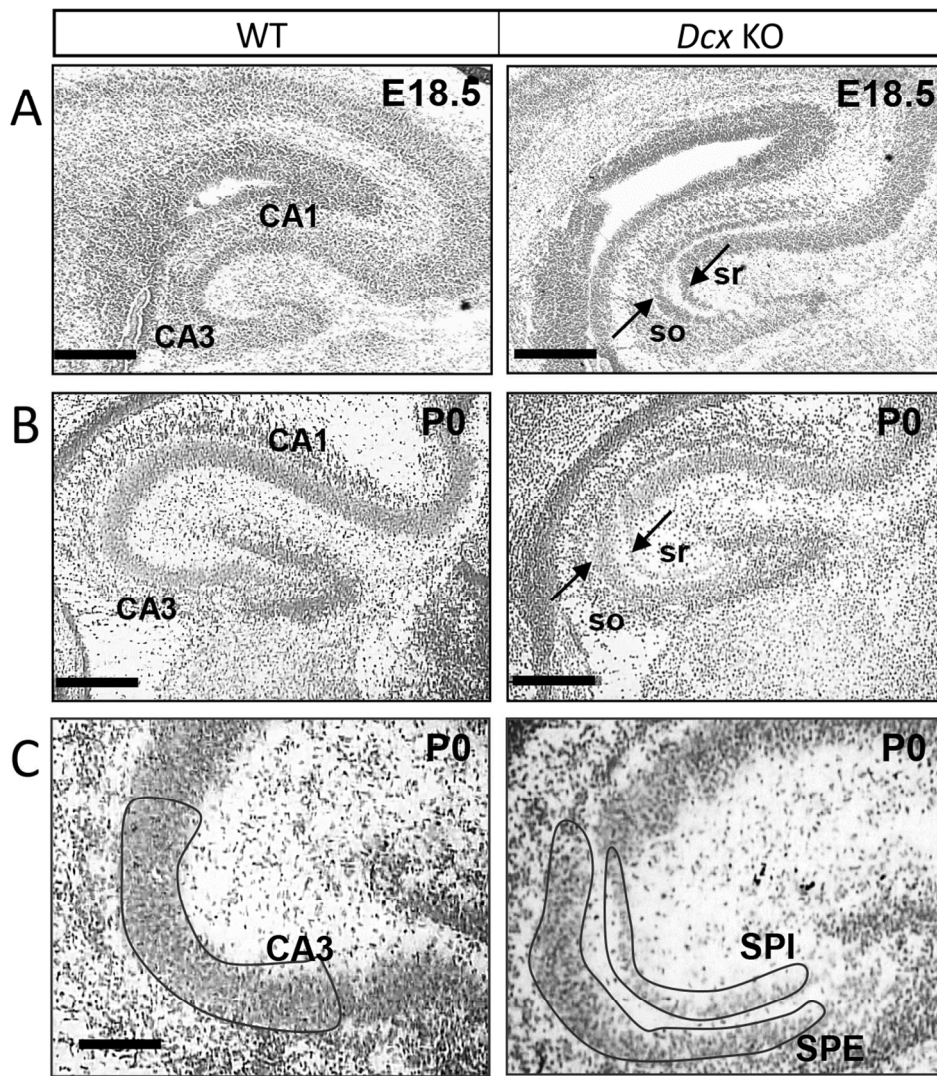
GDPs- giant depolarizing potentials

GABA- gamma-aminobutyric acid

PFA- paraformaldehyde

PBS- phosphate buffered saline

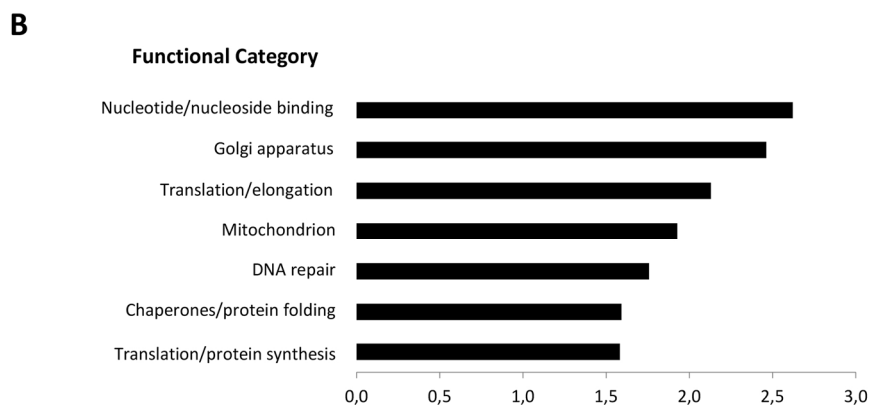
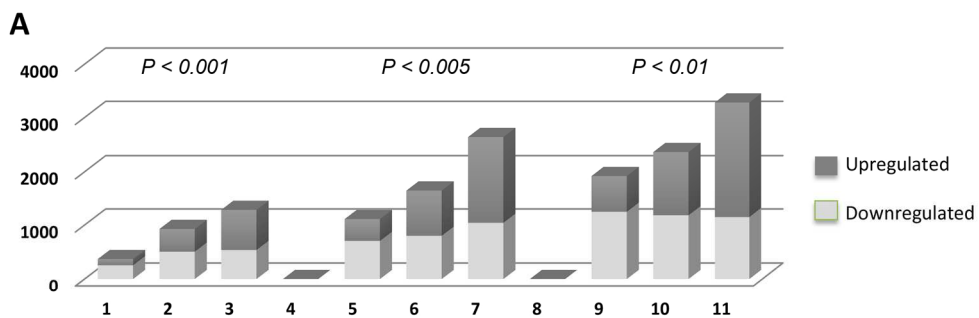
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Khalaf_Stouffer_Fig1

111x122mm (300 x 300 DPI)

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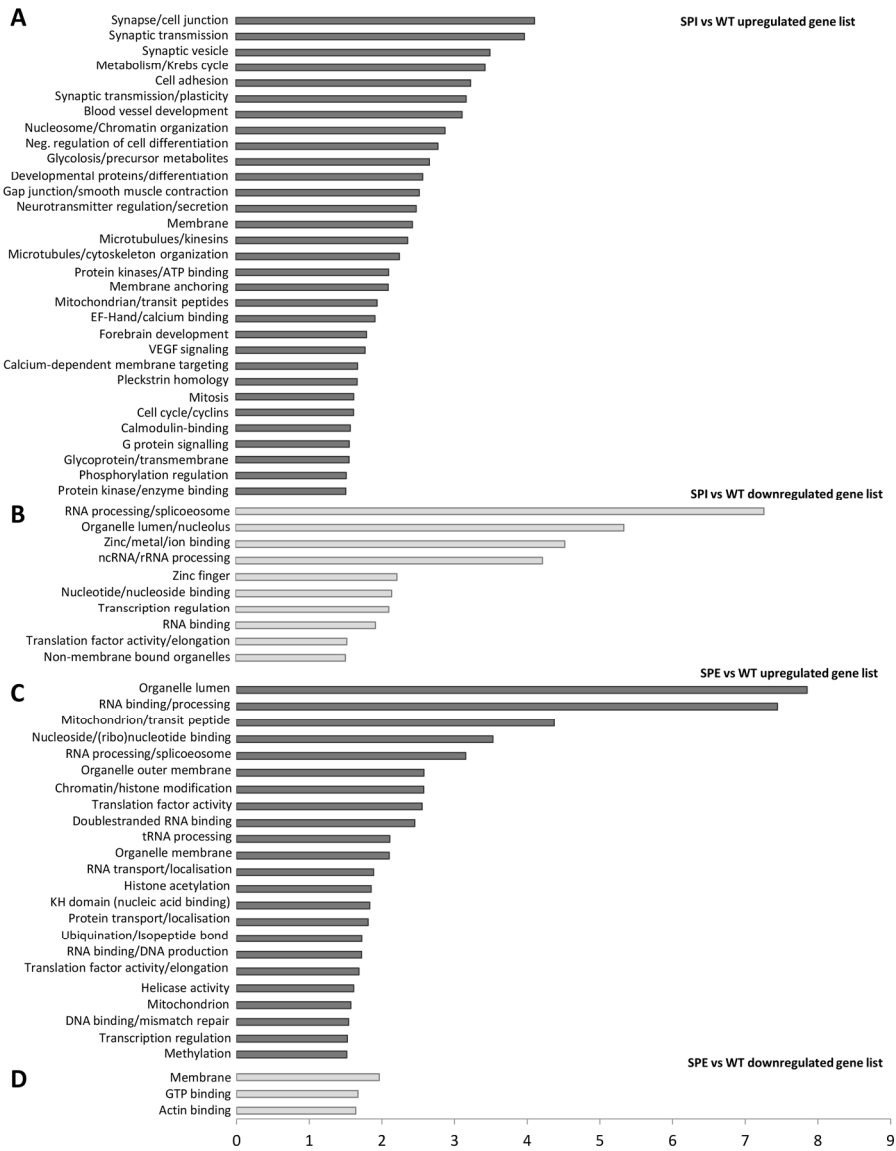
Khalaf_Stouffer_Fig2

173x135mm (300 x 300 DPI)

Review

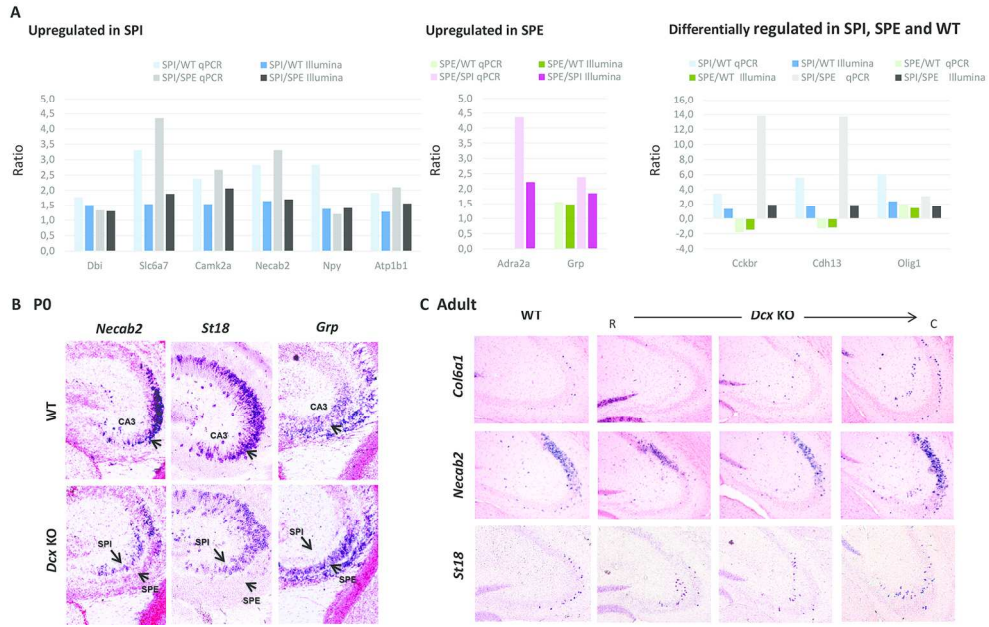
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Figure 3. DAVID functional clustering of SPE and SPI layer-specific gene expression differences.



Khalaf_Stouffer_Fig3

173x228mm (300 x 300 DPI)



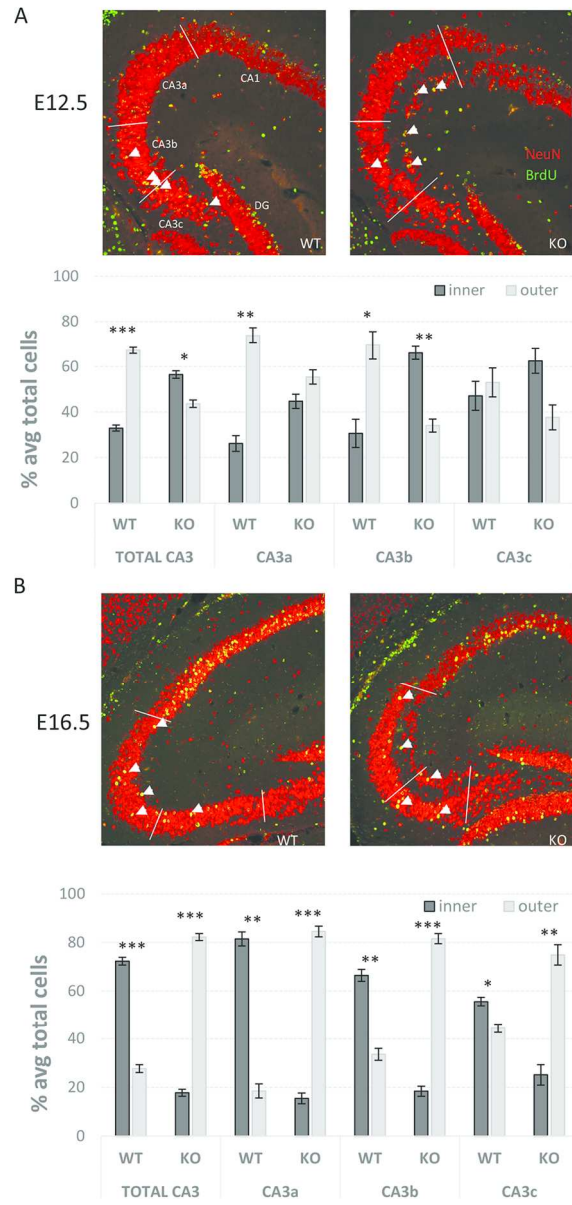
Khalaf_Stouffer_Fig4

173x108mm (300 x 300 DPI)

Review

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Khalaf_Stouffer_Fig5

86x173mm (300 x 300 DPI)